

Rapid and Reversible Reduction of Junctional Permeability in Cells Infected with a Temperature-sensitive Mutant of Avian Sarcoma Virus

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ABSTRACT The transformed or normal phenotype of cultured normal rat kidney cells infected with a temperature-sensitive mutant of avian sarcoma virus is conditional on the temperature at which the cells are grown. Using dye injection techniques, we show that junction-mediated dye transfer is also temperature-sensitive. The extent and rate of transfer between infected cells grown at the transformation-permissive temperature (35°C) is significantly reduced when compared to infected cells grown at the nonpermissive temperature (40.5°C) or uninfected cells grown at either temperature. Infected cells subjected to reciprocal temperature shifts express rapid and reversible alterations of dye transfer capacities, with responses evident by 15 min and completed by 60 min for temperature shifts in either direction. These results suggest that altered junctional capacities may be fundamental to the expression of the ASV-induced, transformed phenotype.

Most cells possess permeable junctions which connect the interiors of adjacent cells and allow the direct exchange of low molecular weight substances (for review, see references 1–3). When present, such junctions provide the capacity for direct electrical communication between electrically excitable cells (4), but their physiological role in electrically nonexcitable cells is not known. One long-standing and somewhat controversial theory states that permeable junctions establish a direct communication pathway for growth-regulating signals and therefore play a crucial role in density-dependent growth regulation (5–7). An important corollary of the theory is that cells with defective junctions are no longer growth regulated and will continue to proliferate in a density-independent fashion (i.e., are transformed).

Experimental evidence for a role of permeable junctions in the process of transformation, obtained primarily from studies of cultured cell lines, has been mixed (e.g. 2, 8–13). By and large, both positive and negative findings are subject to interpretations other than those germane to the possible relationship of reduced junctional communication and transformation. Comparisons between normal and transformed cell lines are difficult at best because of the ambiguities associated with using independently maintained cell lines, which, for example, may contain junctions that are differentially sensitive to a particular culturing regimen (e.g., reference 14). In addition, electrophysiological measurements of cell-to-cell coupling, al-

though ideally suited for detecting the presence or absence of permeable junctions, are less suitable for detecting subtle changes of junctional permeability between well-coupled cells (15). Most importantly, it has not been possible to define the point at which junctions may become altered as the cells acquire the transformed phenotype, nor whether the junctional modification is a prerequisite for transformation.

Many of these problems can be circumvented by using larger junctional permeants than the current-carrying inorganic ions (2) and a cell system in which the transformed or normal state of the cells can be readily reversed. To this end, we have investigated the junctional transfer characteristics of the fluorescent dye Lucifer Yellow CH, using cultured normal rat kidney (NRK) cells infected with a temperature-sensitive mutant of avian sarcoma virus (ASV). These cells are transformed when grown at transformation-permissive temperatures (33–37°C) and normal when grown at nonpermissive temperatures (39.5–40.5°C) (16, 20). Selection of the NRK cell line as a model system was prompted by a report that the ASV gene product responsible for transformation can be localized to the plasma membrane of infected NRK cells, particularly in the vicinity of presumptive gap junctions (17), which are at least one of the structural counterparts of permeable junctions (1).

We have found that the capacity to transfer dye through permeable junctions is substantially reduced when the ASV-infected cells are maintained at the transformation-permissive

temperature. Furthermore, kinetic analyses show that the junctional response to reciprocal temperature shifts is rapid and reversible.

MATERIALS AND METHODS

Cell Cultures

NRK cells containing the proviral form of LA25, a temperature-sensitive mutant of ASV (Prague A strain) (18), were obtained from D. Boettiger (University of Pennsylvania) and A. Faras (University of Minnesota). The cells were originally infected and isolated in the laboratory of P. Vogt (University of California). Uninfected NRK cells were obtained from D. Boettiger. Stock cultures were nurtured in Dulbecco's minimal essential medium (DMEM) containing 10% bovine calf serum, glutamine, and antibiotics; the cells were maintained at 37°C under an atmosphere of 5% CO₂/95% air. Cells to be injected were plated onto 60-mm plastic cultures dishes at a density of $1-2 \times 10^5$ cells/dish and grown in the above growth medium/CO₂ atmosphere at either 35°C or 40.5°C for 1-3 d.

Fluorescent Dye

Lucifer Yellow CH was synthesized and generously provided by W. Stewart (National Institute of Arthritis, Metabolic, and Digestive Diseases). The characteristics of the dye have been described (19). Briefly, the molecule is a highly fluorescent naphthalimide dye with two sulfonic acid groups yielding a net charge of negative two. Attributes of the dye, in addition to intense fluorescence, include an inability to cross nonjunctional membrane, rapid spread within the cell, and, with a molecular weight of 457, a sufficiently small size to pass through junctional channels.

Dye Injection

Dye injection was accomplished using 3-6 nA of direct current pulses applied through a glass microelectrode containing a 4% aqueous solution of Lucifer Yellow CH. Duration of the negative current pulse was 350 ms at a frequency of 2/s. Test cells were rinsed three times with pre-warmed (37°C) phosphate-buffered saline (PBS, pH 7.3), and maintained in a grounded PBS bath at room temperature during dye injection. A limited number of injections were performed on LA25-NRK cells maintained at their growth temperature—40.5°C or 35°C. The magnitude of the differences in dye transfer capacities between transformed and normal cells was indistinguishable from that obtained with cells microinjected at room temperature. Approximately 6 min were required for the PBS bath to reach room temperature (20-21°C). The observations of dye spread and measurements of transfer-time intervals (see below) indicated that junctional dye transfer is constant for at least 50 min when the cells are maintained in PBS at room temperature. All microinjections were performed within 40 min for each plate of cells, in which time four to eight penetrations were typically obtained. For temperature shift experiments, cells maintained at 40.5°C or 35°C for at least 12 h were shifted to 35°C or 40.5°C (without changing the growth medium) for 15, 30, or 60 min. The cells were then rinsed three times with warmed PBS and microinjected as described above.

Microscopy

Impalement of a cell and subsequent transfer of dye to neighboring cells was monitored with a Leitz dark-field fluorescence microscope equipped with Blau excitation filter BG 12, heat-absorbing filter BG 38, and a blue-absorbing barrier filter. The extent of dye spread was photographically recorded on Kodak Tri-X film with 1-min exposures. Typically, dye was injected for 3 min and the exposures were taken 2 min later. Over the 3-min injection period, dye could be seen to pulse into the injected cell, ensuring that a continuous delivery was obtained, but, because of electrode variability, it was not possible to deliver exactly the same amount of dye from experiment to experiment. Nevertheless, the standard errors from Tables I and II suggest that electrode variability does not appreciably affect the results.

The extent of dye spread obtained after 5 min represents an integrated form of the underlying rate at which dye is being transferred. To provide a more direct measure of the rate of dye transfer, initial rates of dye transfer were estimated by measuring the time elapsed from initiation of dye injection into a cell surrounded on all sides by neighboring cells to detection of fluorescence in any one of the adjacent cells. These measurements provided a more quantitative basis for comparing the junctional transfer characteristics of normal and transformed cells. To avoid inferences that an actual rate was being measured, the estimates have been termed "transfer-time intervals."

RESULTS

Junctional dye transfer between uninfected NRK cells is always extensive (Fig. 1 *e* and *f*), more so than that typically obtained

with other established cell lines. All cells within the boundary of dye spread accumulate dye, including rounded mitotic cells. The extent of dye transfer between LA25-NRK cells, however, depends on the temperature at which the cells are grown. Infected cells grown at the nonpermissive temperature (40.5°C) invariably transfer dye to a greater extent (Fig. 1 *a*) than infected cells grown at the permissive temperature (35°C) (Fig. 1 *c*). Although severely restricted, junctional dye transfer between LA25-NRK cells grown at 35°C is always obtained, indicating that the transformed cells do not completely "uncouple."

Uninfected NRK cells grown at either 40.5°C or 35°C show extensive and comparable transfer capabilities (Fig. 1 *e* and *f*), suggesting that the pronounced reduction of dye transfer between permissively grown LA25-NRK cells is not a consequence of the growth temperature per se.

Transfer-time Intervals

To more precisely define the differences in dye spread between normal and transformed NRK cells, junctional transfer was further evaluated by measuring the time elapsed from the beginning of dye injection into the impaled cell to detection of fluorescence in an adjacent cell ("transfer-time interval" - see Materials and Methods). As shown in Table I, the values obtained are consistent with the observations of dye spread presented above. Uninfected NRK cells grown at 35°C or 40.5°C exhibit equivalent average transfer-time intervals, which are comparable to the value of LA25-NRK cells grown at 40.5°C. On the other hand, LA25-NRK cells grown at 35°C require considerably more time to exchange dye with neighboring cells. Comparison of the transfer-time intervals suggests that the junctional capacity of LA25-NRK cells is reduced ~70% upon transformation.

Because of the parameters involved in obtaining transfer-time intervals, the 70% value is probably a conservative estimate. Measurements of the transfer-time interval include not only the actual transit time of dye passage through junctional channels but also the time required for dye to reach a junctional interface and accumulate to detectable levels in an adjacent cell. In addition, the estimates are influenced by the concentration gradient of dye established across the junctional interface. As shown in Fig. 2, LA25-NRK cells grown at 35°C appear to be smaller and more rounded than their counterparts grown at 40.5°C, in effect reducing the distance between the point of dye injection and the cell periphery. Moreover, a rounded cell will produce a higher intensity of fluorescence than a flat cell containing an equivalent concentration of dye. Thus, both dye spread to junctional interfaces and detection of dye within a recipient cell should involve less time for 35°C cells. Most importantly, transfer is detected only after 26 s on average for the transformed cells, at which time the continuous injection of dye has established a larger concentration gradient across the junctional interface than that obtained by 7 s on average for the 40.5°C cells. All of these factors combine to produce a probable underestimate of the actual differences in junctional transfer capabilities.

Kinetics of the Junctional Response

An indication of the relationship between LA25-induced transformation and reduced junctional capacity can be obtained by determining the rate at which the junctions respond to acute temperature shifts. A rapid change in junctional transfer capacity would suggest that the junctional modification is less likely a peripheral response to the events required for

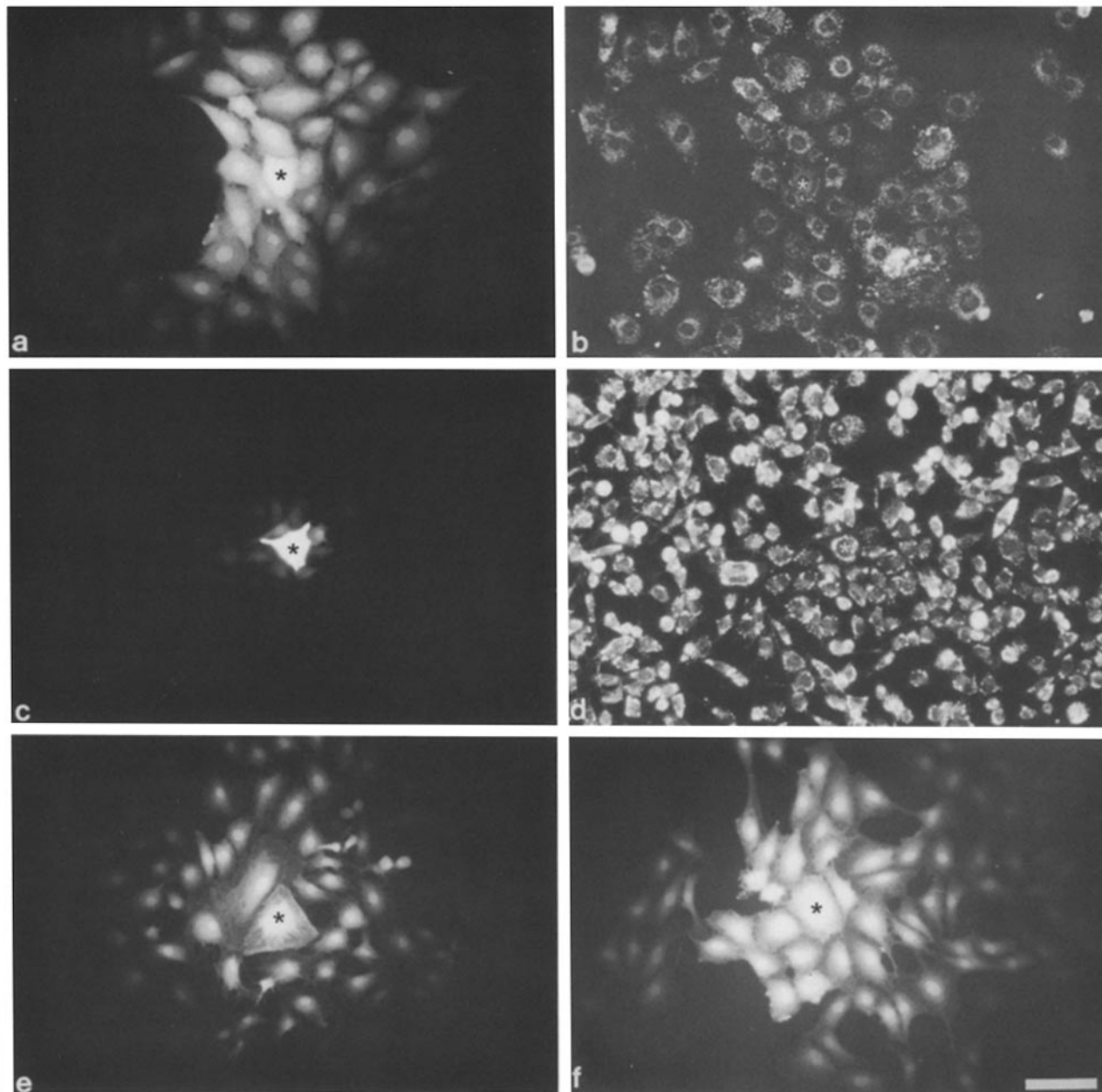


FIGURE 1 Micrographs showing the extent of dye spread in LA25-NRK cells and uninfected NRK cells grown at 40.5°C or 35°C. Asterisks indicate injected cells. *a*, LA25-NRK cells grown at 40.5°C. *b*, dark-field micrograph of *a*. *c*, LA25-NRK cells grown at 35°C. *d*, dark-field micrograph of *c*. *e*, uninfected NRK cells grown at 40.5°C. *f*, uninfected NRK cells grown at 35°C. For each condition, the cell was injected with Lucifer Yellow CH for 3 min and photographed 2 min later. Bar, 100 μm . $\times 110$.

transformation. Typical patterns of dye spread in response to temperature shifts (see Materials and Methods) are shown in Fig. 3. (For purposes of comparison, the cells shown in Fig. 3 were from replicate plates of the cells shown in Fig. 1.) Dye spread between LA25-NRK cells grown at 40.5°C and shifted to 35°C 30 min before dye injection (Fig. 3*a*) is substantially less extensive than that obtained from a replicate plate grown at 40.5°C and not subjected to temperature downshift (Fig. 1*a*). Similarly, by 30 min of temperature upshift from 35°C to 40.5°C, LA25-NRK cells display an increased capacity to transfer dye (Fig. 3*c*) relative to unshifted 35°C cells from a replicate plate (Fig. 1*c*). Note that the morphology of the cells grown at 40.5°C does not appear to change after 30 min at 35°C (compare Figs. 1*b* and 3*b*), nor are morphological differences apparent after 30 min of temperature upshift (compare

Figs. 1*d* and 3*d*). We have found that fully developed morphology differences such as those shown in Fig. 2 require ~ 8 h or more of further incubation, as has been reported by others (20).

To determine whether the junctional response to temperature downshift can be reversed, LA25-NRK cells grown at 40.5°C were shifted to 35°C for 60 min, dye injected (Fig. 4*a*), and then placed back at 40.5°C for an additional 60 min. As shown in Fig. 4*c*, injecting a cell with dye after the second incubation produced widespread dye transfer, indicating that the response is reversible and that the experimental procedure for dye injection does not adversely affect the cells. Again, morphological differences are not apparent after 60 min at 35°C (Fig. 4*b*) or 40.5°C (Fig. 4*d*).

Measurements of the transfer-time intervals following tem-

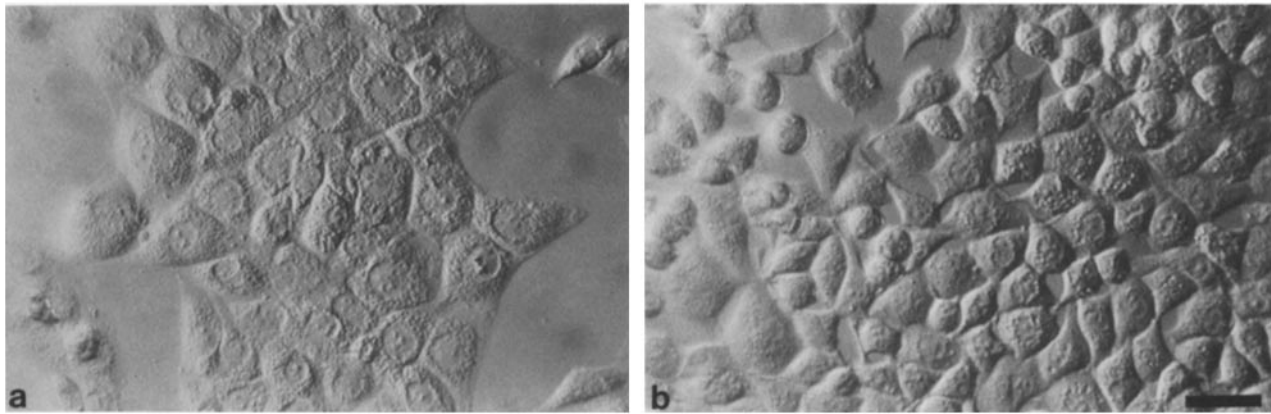


FIGURE 2 LA25-NRK cells grown at 40.5°C (a) or 35°C (b). Nomarski optics of live cells in PBS at room temperature. Bar, 50 µm. × 200.

TABLE I
Average Transfer-time Intervals of Cells Maintained at 35°C or 40.5°C*

| Cell line | Growth temperature °C | Average transfer-time interval ± SEM s | Number of penetrations |
|----------------|--------------------------|--|------------------------|
| LA25-NRK | 35 | 26.1 ± 3.4 | 35 |
| LA25-NRK | 40.5 | 6.6 ± 0.4 | 35 |
| Uninfected NRK | 35 | 5.8 ± 0.5 | 14 |
| Uninfected NRK | 40.5 | 5.7 ± 0.8 | 11 |

* Data for the average transfer-time intervals were obtained from 3–6 separate experiments.

perature shifts demonstrate more precisely the rapidity of the junctional response (Table II). Changes in the average transfer-time intervals are evident as early as 15 min after downshift to the permissive temperature or upshift to the nonpermissive temperature. The values appear to plateau by 30 min for temperature upshifts, and by 60 min for temperature downshifts. At these times, the average transfer-time intervals of cells downshifted to 35°C or upshifted to 40.5°C approximate the average transfer-time intervals of nonshifted cells grown at 35°C or 40.5°C, respectively. These results suggest that the influence of cell morphology on the estimates of transfer-time intervals is relatively minor, since transfer-time intervals typical of nonshifted cells are attained at a time when shape changes are not apparent. However, morphological considerations may help explain the observation that the transfer-time intervals plateau in 30 min with temperature upshifts vs. 60 min for temperature downshifts. That is, cells shifted from 35°C to 40.5°C maintain the morphology more favorable for early detection of dye spread to an adjacent cell, and vice versa for shifts from 40.5°C to 35°C (see above).

DISCUSSION

Our studies demonstrate that NRK cells infected with a mutant strain of ASV that is temperature-sensitive for transformation are also temperature-sensitive with respect to junction-mediated dye transfer. The extent of dye transfer between infected cells maintained at the transformation-permissive temperature is significantly reduced when compared to that between infected cells maintained at the nonpermissive temperature; measurements of the transfer-time intervals suggest that the junctional capacity for dye transfer is reduced ~70%. We believe that three observations deserve special emphasis: (a) As previ-

ously discussed (see Results), the temperature shift experiments suggest that this reduction cannot be attributed to changes in cell morphology. This is further supported by the observation that changes in the immunofluorescence pattern of actin filaments in LA25-NRK cells is first consistently observed only by 1.5 h after temperature downshift.¹ (b) Uninfected NRK cells transfer dye equally well when grown at 40.5°C or 35°C, indicating that the junctions of NRK cells are not intrinsically sensitive to these growth temperatures. (c) Temperature shifts in either direction produce an extremely rapid change in the dye-transfer capacities of infected cells. Responses are detectable by 15 min and essentially complete by 60 min, implying that the temperature-sensitivity of dye transfer may be directly related to the temperature-sensitive mechanisms responsible for transformation of LA25-NRK cells.

The junctional response of LA25-NRK cells presumably reflects modification of the number and/or effective bore size of junctional channels. Since formation of gap junctions can occur within the time frame of the junctional response to temperature shifts (21), it is conceivable that the increase of junctional permeability following upshift to 40.5°C is due to formation of new junctions. Similarly, loss of gap junctions (e.g., endocytosis [22] or possibly dispersion of gap-junctional particles) might account for the reduction of permeability following downshift to 35°C. We have conducted preliminary freeze-fracture studies of gap junctions in transformed and normal LA25-NRK cells. The results suggest that differences in the size of junctions and the ordering of intramembrane junctional particles do exist (S. K. Anderson et al., abstract submitted for publication). However, the rate at which these differences are manifest following temperature shifts is slower than the permeability changes.

Since pp60^{src} is responsible for transformation of ASV-infected cells (for review, see reference 23), the possibility exists that the junctional response of LA25-NRK cells to transformation-permissive conditions involves a direct interaction between pp60^{src} and junctional components. This possibility is strengthened by our observation that the junctional response to temperature downshift is quite rapid, corresponding well to the appearance of an active pp60^{src}-associated protein kinase demonstrated in other cell systems (24, 25). Moreover, recent reports suggest that a variable proportion of total cellular

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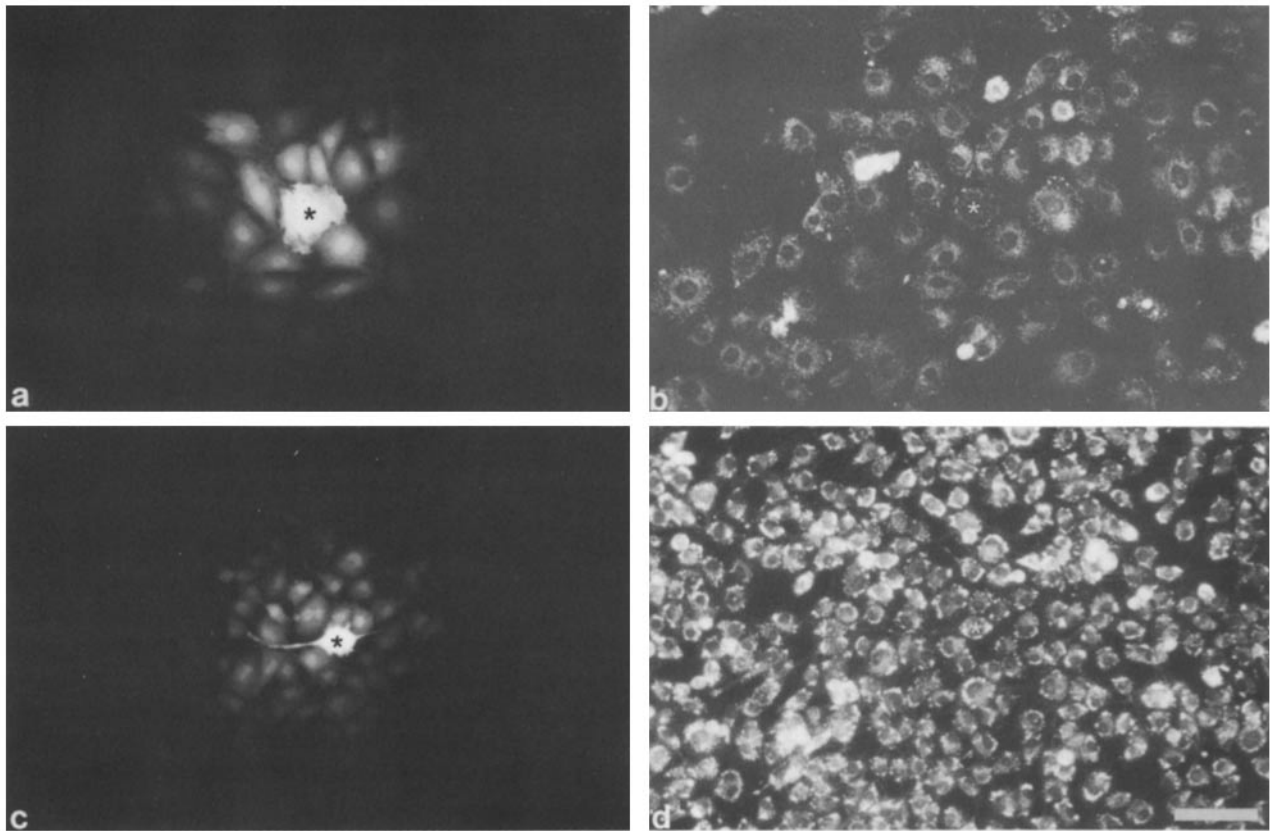


FIGURE 3 Extent of dye spread in LA25-NRK cells following temperature shifts. Asterisk indicates injected cell. *a*, LA25-NRK cells grown at 40.5°C and shifted to 35°C for 30 min before injection. *b*, dark-field micrograph of *a*. *c*, LA25-NRK cells grown at 35°C and shifted to 40.5°C for 30 min before injection. *d*, dark-field micrograph of *c*. For each condition, the cell was injected for 3 min and photographed 2 min later. Bar, 100 μm . $\times 110$.

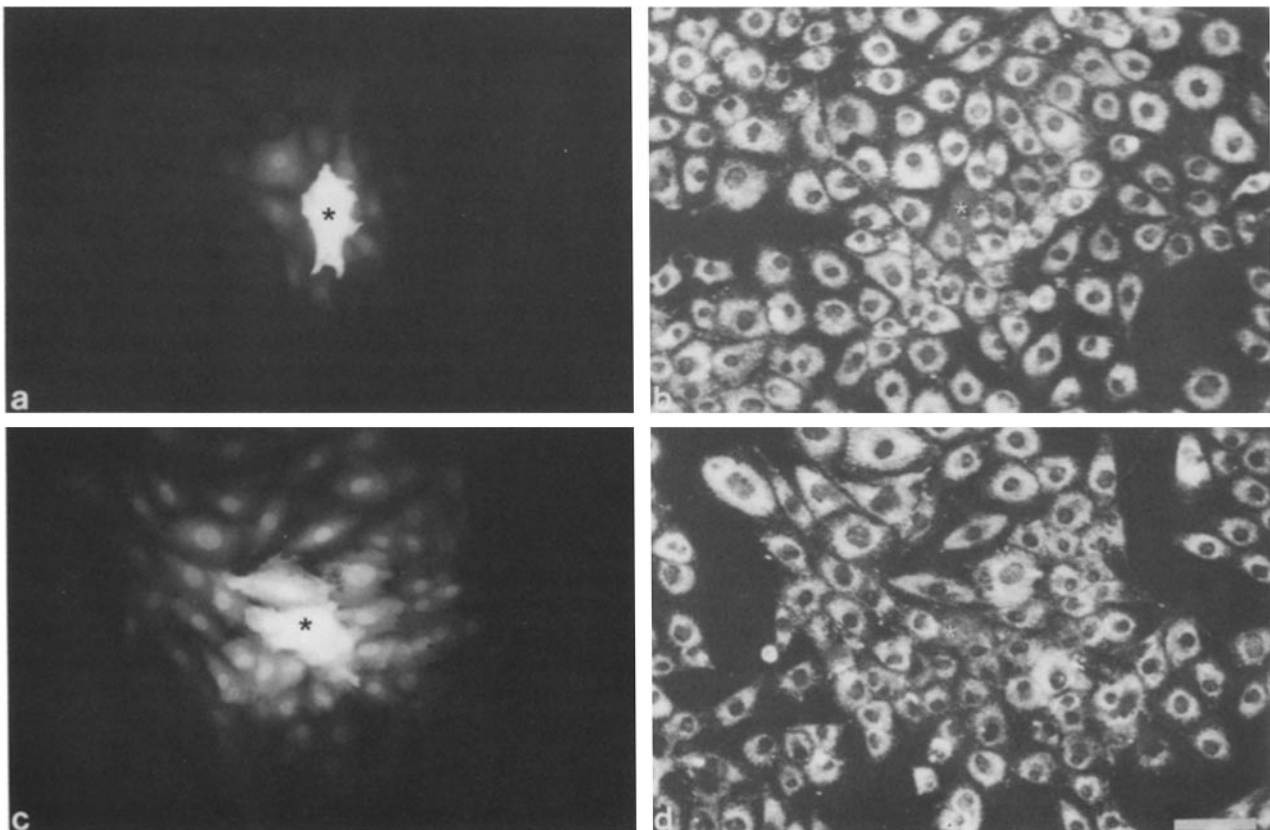


FIGURE 4 LA25-NRK cells subjected to reciprocal temperature shifts. Asterisk indicates injected cell. *a*, cells grown at 40.5°C and shifted to 35°C for 60 min before dye injection. *b*, dark-field micrograph of *a*. *c*, the same dish of cells was subsequently shifted up to 40.5°C (in growth medium) for 60 min before dye injection. *d*, dark-field micrograph of *c*. For each condition, the cell was injected for 3 min and photographed 3 min later. Bar, 100 μm . $\times 110$.

TABLE II
Transfer-time Intervals from LA25-NRK Subjected to Reciprocal Temperature Shifts*

| Direction of shift | Time of incubation | Average transfer-time interval \pm SEM | Number of penetrations |
|---------------------------|--------------------|--|------------------------|
| | min | s | |
| 40.5°C \rightarrow 35°C | 0 | 6.6 \pm 0.4 | 35 |
| | 15 | 13.9 \pm 1.5 | 19 |
| | 30 | 18.0 \pm 1.6 | 23 |
| | 60 | 24. \pm 3.6 | 15 |
| 35°C \rightarrow 40.5°C | 0 | 26.1 \pm 3.4 | 35 |
| | 15 | 14.9 \pm 1.8 | 24 |
| | 30 | 6.6 \pm 0.4 | 20 |
| | 60 | 6.2 \pm 0.5 | 10 |

* Data for the average transfer-time intervals were obtained from 3-6 separate experiments.

pp60^{src} is associated with the plasma membrane (26, 27), and an immunocytochemical study suggests that pp60^{src} is concentrated in the vicinity of gap junctions in ASV-infected NRK cells (17). Alternatively, it is conceivable that the linkage between expression of pp60^{src} activity and reduced junctional permeability requires a rapidly occurring intermediate step, possibly involving an increase in intracellular free calcium or a reduction in intracellular pH, both of which have been reported to rapidly and reversibly reduce junctional permeability (28, 29).

If a general effect of pp60^{src} is to reduce junctional permeability, then transformation of any cell line by ASV should result in a reduction of junctional permeability. O'Laque and Dalen (9) reported that cultured chick embryo cells infected with ASV are electrically coupled (which is consistent with our observations of dye spread in LA25-NRK cells grown at the transformation-permissive temperature). However, the range in the degree of electrical coupling did not appear to be different from that measured in uninfected cells. The question remains whether junctional differences not detected by electrical measurements may be demonstrable using dye injection techniques on ASV-transformed chick embryo cells, and whether ASV-infected cell systems other than LA25-NRK contain impaired permeable junctions. We have conducted preliminary studies which suggest that junctional dye transfer is reduced in European field vole fibroblasts infected with wild-type ASV (Schmidt-Ruppin strain), and in NRK cells infected with LA29 (another temperature-sensitive mutant of ASV) grown at the transformation-permissive temperature.

The results presented here strongly suggest that the phenomena of reduced junctional permeability and transformation of LA25-NRK cells are interrelated. To the extent that early responses to an active transforming gene product represent those processes most likely to be critically involved in producing the transformed phenotype, the rapid junctional response of LA25-NRK cells to transformation-permissive conditions implicates altered junctional permeability as one of those requisite processes of ASV-induced transformation.

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